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Accumulation of Zeaxanthin in Abscisic Acid-Deficient Mutants of Arabidopsis Does Not Affect Chlorophyll Fluorescence Quenching or Sensitivity to Photoinhibition in Vivo

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Abscisic acid (ABA)-deficient mutants of Arabidopsis do not synthesize the epoxy-xanthophylls antheraxanthin, violaxanthin, or neoxanthin. However, thylakoid membranes from these mutants contain 3-fold more zeaxanthin than wild-type plants. This increase in zeaxanthin occurs as a stoichiometric replacement of the missing violaxanthin and neoxanthin within the pigment-protein complexes of both photosystem I and photosystem II (PSII). The retention of zeaxanthin in the dark by ABA-deficient mutants sensitizes the leaves to the development of nonphotochemical quenching (NPQ) during the first 2 to 4 min following a dark-light transition. However, the increase in pool size does not result in any increase in steady-state NPQ. When we exposed wild-type and ABA-deficient mutants leaves to twice growth irradiance, the mutants developed lower maximal NPQ but suffered similar photoinhibition to wild-type, measured both as a decline in the ratio of variable to maximal fluorescence and as a loss of functional PSII centers from oxygen flash yield measurements. These results suggest that only a few of the zeaxanthin molecules present within the light-harvesting antenna of PSII may be involved in NPQ and neither the accumulation of a large pool of zeaxanthin within the antenna of PSII nor an increase in conversion of violaxanthin to zeaxanthin will necessarily enhance photoprotective energy dissipation.

Photoinhibition is the light-dependent loss of photosynthetic efficiency that occurs when the light-harvesting antennae absorb more excitation energy than is dissipated by photochemistry (Powles, 1984; Osmond, 1994). In the field, the point at which excitation capture exceeds the capacity for electron withdrawal from PSII may vary widely, with the effects of fluctuations in temperature, water, and nutrient status superimposed on fluctuations in irradiance. Higher plants have evolved a range of mechanisms that enable them to avoid photodamaging reactions. These include both fast-avoidance mechanisms, such as changes in leaf and chloroplast orientation (reviewed by Björkman and Demmig-Adams, 1994), and long-term acclimative responses, such as modulation of thylakoid composition (Anderson and Osmond, 1987) and changes to leaf reflectance (Robinson et al., 1993). However, such mechanisms either require a period of acclimation or are not universal. Thus, much attention has focused on the apparently ubiquitous energy dissipative processes present in higher plant thylakoids that allow for rapid down-regulation of PSII energy capture efficiency under excess irradiance.

NPQ is thought to reflect the activity of a range of mechanisms associated with both the PSII reaction center and the light-harvesting antenna of PSII, which act to dissipate excess excitation energy safely as heat. The principal driving force for these dissipative reactions is the development of the thylakoid pH gradient (Briantais et al., 1979; Krause et al., 1982; Gilmore et al., 1995). Both reaction center and antenna-based mechanisms have been implicated in these dissipative reactions. Lumen acidification has been shown to slow electron donation from the oxygen-evolving complex to $P680^+$ (Krieger and Weis, 1990). Under these conditions, functional PSII reaction centers can dissipate trapped excitation energy nonphotochemically by fast internal charge recombination of the $P680^+Pheo^-$ radical pair (Schlodder and Brettel, 1988; Krieger and Weis, 1990). Quenching of Chl *a* fluorescence by photoinhibited PSII reaction centers is thought to involve impaired charge separation (Eckert et al., 1992). Quenching could be enhanced in these photoinhibited reaction centers by either changes in the rate constants for

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Abbreviations: Chl, chlorophyll; F_m and F_m', maximal fluorescence when all PSII reaction centers are closed in dark- and light-acclimated leaves, respectively; F_o and F_o', minimal fluorescence when all PSII reaction centers are open in dark- and light-acclimated leaves, respectively; F_s, fluorescence at steady state; F_v, variable fluorescence ($F_m - F_o$) in dark-acclimated leaves; F_v', variable fluorescence ($F_m' - F_o'$) in light-acclimated leaves; LHC, light-harvesting complex; NPQ, nonphotochemical quenching of Chl a fluorescence; P680, primary electron donor of the reaction center of PSII; PFD, photosynthetic flux density; q_P, photochemical quenching of fluorescence.

charge stabilization and recombination or by the generation of a Chl^+ radical close to $P680^+$ (Renger et al., 1995). However, these mechanisms are still under investigation, and it remains to be determined how much energy they could dissipate in vivo.

Antenna quenching, which may reduce the flux of excitation energy into PSII when the capacity for electron withdrawal from the reaction center is limiting, is purported to be the primary nonradiative energy dissipating mechanism in higher plant thylakoids (Demmig-Adams and Adams, 1992). Acidification of the thylakoid lumen stimulates the activity of violaxanthin de-epoxidase, shifting the balance between the epoxidase and de-epoxidase reactions and favoring the accumulation of zeaxanthin (Yamamoto et al., 1962; Siefermann and Yamamoto, 1974; Yamamoto, 1979). A low luminal pH also results in the protonation of carbonyl residues at the luminal surface of the light-harvesting pigment-protein complexes (Ruban et al., 1993). This has led to the proposal that the combination of zeaxanthin accumulation and protonation of the antenna proteins leads to the formation of aggregated LHCIIb complexes that facilitate nonradiative decay (Ruban et al., 1992, 1993). Alternatively, the minor Chl *a/b*-binding proteins (LHCIIa, c, and d) are enriched in violaxanthin (Bassi et al., 1993; Ruban et al., 1994). This finding and data from *chlorina* barley mutants and intermittent-light-grown plants lacking LHCIIb have led to the suggestion that the minor Chl *a/b*-binding proteins may be the primary site of xanthophyll-related energy dissipation in the antenna of PSII (Bassi et al., 1993; Crofts and Yerkes, 1994; Jahns and Schweig, 1995; Gilmore et al., 1996b). Recent studies using time-resolved Chl *a* fluorescence also suggest that nonphotochemical energy dissipation is related to the binding of de-epoxidized xanthophylls (A + Z) to the inner PSII antenna (Gilmore et al., 1996a). However, the primary site of antenna quenching and the degree to which such quenching units can divert energy from PSII has not been established unequivocally.

The ABA-deficient mutants of Arabidopsis (*aba1–3* and *aba1–4*) are unable to synthesize the epoxy-xanthophylls neoxanthin, antheraxanthin, and violaxanthin, and of the xanthophylls only lutein and zeaxanthin are present (Duckham et al., 1991; Rock and Zeevaart, 1991). In this study we tested the hypothesis that increasing the zeaxanthin content of the PSII light-harvesting antenna increases the quenching capacity of these complexes in the ABA-deficient mutants and alters the sensitivity of the leaves to photoinhibition. A secondary aim of these experiments was to determine what effect the absence of the epoxy-xanthophylls violaxanthin and neoxanthin has on the composition and function of the light-harvesting antennae.

MATERIALS AND METHODS

Seeds of *Arabidopsis thaliana* L. (Heynh.), ecotype Landsberg *erecta* (wild type) and the mutant genotypes *aba1–3* and *aba1–4* (isolation G4 and A73, respectively [Koornneef et al., 1982]) were obtained from the Arabidopsis Biological Resource Center (Ohio State University, Columbus). Wild-type seeds were planted directly onto a peat:sand:compost (3:7:3, w/v) mixture and covered with plastic wrap for 7 d. The *aba1–3* and *aba1–4* mutants were first germinated on 1% agar containing 5 μM ABA for 4 d and then germinated seeds were transplanted onto a peat:sand:compost (3:7:3, w/v) mixture and covered with plastic wrap for 7 d. All plants were grown in a high-humidity (90%) growth chamber and maintained on a diurnal cycle of 10 h of light (500 μmol m^{-2} s^{-1} PFD) at 21°C and 14 h of dark at 18°C. Plants were watered as required with one-half-strength Hoagland solution. Leaves from 28- to 35-d-old plants were used for all experiments.

Membrane Isolation and Nondenaturing Green Gel Electrophoresis

Thylakoid membranes were isolated rapidly at 4°C after homogenization of the leaves for 5 s in a 50 mM Mes-NaOH grinding buffer (pH 6.5) containing 5 mM Mg_2Cl, 10 mM NaCl, 0.2% BSA, 0.2% L-ascorbic acid, 1% polyvinylpolypyrrolidone, and 400 mM sorbitol. The brei was filtered through three layers of Miracloth (Calbiochem) and centrifuged at 5000*g* for 5 min at 4°C. The pellet was resuspended in a 50 mM Mes-NaOH (pH 6.5) wash buffer containing 5 mM Mg_2Cl, 10 mM NaCl, and 400 mM sorbitol and centrifuged at 5000*g* for 1 min. The pellet was then resuspended in a minimal volume of the wash buffer prior to determination of the Chl content.

Thylakoid membranes were solubilized by adding 10 μL of the thylakoid resuspension (1 μg Chl μL^{-1}) to 20 μL of a 15 mM Tris (pH 8.3) sample buffer containing 120 mM Gly to which 10 μL of 10% (w/v) decyl-β-*d*-maltoside (Calbiochem) was added, giving a detergent:Chl ratio of 100:1 (v/v). The thylakoids were solubilized at 4°C for 60 min and then centrifuged briefly in a microfuge prior to electrophoresis at 4°C on 8% polyacrylamide gels (Lee and Thornber, 1995). Electrophoresis was carried out in 12 mM Tris (pH 8.3) with the top buffer containing 0.01% SDS and 0.2% Deriphat (Henkel, Hoboken, NJ). To obtain sufficient pigments for analysis, eight small tube gels (5 mm in diameter) were loaded with 8 μg of Chl per gel and run simultaneously.

The gels were scanned twice at 675 and 650 nm with a spectrophotometer (model 635, Varian, Sunnyvale, CA) and the relative distribution of Chl between the resolved bands was estimated by averaging the results of the two scans. The identity of the resolved bands was determined by measuring the absorption spectra with a spectrophotometer (model 557, Hitachi Perkin-Elmer, Tokyo, Japan) and the polypeptide composition was confirmed by SDS-PAGE.

Pigments

For whole-leaf extracts, Chls and carotenoids were measured from three replicate leaf discs (0.78 cm^2) frozen in liquid N_2. The frozen samples were ground with a mortar and pestle and extracted in ice-cold 100% acetone (acetone-water mixtures did not improve carotenoid extraction). Pigments were measured by HPLC and separated on a column (Spherisorb ODS1, Alltech Associates, Sydney,

Australia) at a flow rate of 1 mL min^{-1} with solvents A and B, as described previously (Gilmore and Yamamoto, 1991). The injection volume was 20 μL. The column was calibrated either with standards prepared by TLC (Demmig et al., 1987) (neoxanthin, violaxanthin, antheraxanthin, zeaxanthin, lutein, Chl *a*, and β-carotene) or using commercial preparations (Chl *b*, Sigma). The concentration of Chl was determined spectrophotometrically in 80% buffered acetone (Porra et al., 1989).

To determine the concentrations of pigment in thylakoids, leaves were macerated for 5 s in ice-cold 50 mM Tricine grinding buffer (pH 7.8) containing 400 mM sorbitol, 5 mM $MgCl_2$, and 10 mM NaCl. The brei was filtered through two layers of Miracloth (Calbiochem) and the filtrate was centrifuged at 4,000*g* for 3 min at 4°C. The pellet was washed twice by resuspension in a 50 mM Tricine wash buffer (pH 7.8) containing 10 mM NaCl and 5 mM $MgCl_2$ and centrifuged at 10,000*g* for 5 min at 4°C. The final pellet was then resuspended in a 50 mM Tricine resuspension buffer (pH 7.8) containing 400 mM sorbitol, 10 mM NaCl, and 5 mM $MgCl_2$ and stored on ice in the dark. Aliquots were removed and added to 80% acetone for pigment analysis by HPLC as described above.

The concentration of pigments in the pigment-protein complexes resolved by Deriphat-PAGE was determined after electro-elution (model 422, Bio-Rad) of the pooled excised bands at 4°C using a 25 mM Tris buffer (pH 8.3) containing 192 mM Gly and 0.2% Deriphat. The eluates were concentrated using ultrafree-MC low-binding, regenerated cellulose filters (30,000 nominal molecular weight, Millipore) and made to 80% acetone for HPLC as described above.

Steady-State Oxygen Evolution

Steady-state photosynthetic oxygen evolution was measured with a leaf disc electrode (model LD2, Hansatech, King's Lynn, Norfolk, UK) at 23°C in humidified air containing 5% CO_2. The top of the cuvette was modified to fit the fiberoptic of a pulse-amplitude modulation Chl fluorometer (PAM, Walz, Effeltrich, Germany), and continuous actinic white light was supplied by a Schott lamp (model KL 1500, Schott, Mainz, Germany). The desired PFD, as measured at the position of the leaf by a quantum sensor (model Li-185 A; Li-Cor, Lincoln, NE), was achieved by varying the output from the lamp. The rate of oxygen evolution (dO_2/dt) was calculated using the Hansatech A/D interface board (model IF/1) and the associated "LeafDisc" software installed in an IBM-compatible personal computer.

Chl Fluorescence

Chl *a* fluorescence was measured using a modulated fluorometer (PAM, Walz) with the PAM 103 accessory and two Schott lamps (model KL 1500; Schott), providing saturating flashes (FL 103) and actinic illumination. The experimental protocol for measuring the light response in leaves has been described in detail elsewhere (Hurry et al., 1993). Fluorescence induction kinetics were monitored on attached leaves in air at 23°C and at different PFDs ranging from 10 to 2000 μmol m^{-2} s^{-1}. Fluorescence characteristics were evaluated when the steady-state F_s level was reached, which, depending on PFD, occurred 20 to 40 min after switching to the next higher irradiance. Photochemical quenching, NPQ, WPQ, and the yield of electron transport were calculated as described previously (Genty et al., 1989; Schreiber et al., 1994).

Slow fluorescence transients were monitored in attached leaves in air at 23°C using the fluorometer as described previously (Hurry et al., 1993). Following the determination of F_o, F_v, and F_m in the dark-adapted state, fluorescence quenching parameters were monitored during induction of photosynthesis at an actinic irradiance of either 500 or 1000 μmol m^{-2} s^{-1} PFD with a flashing frequency for the saturating pulse (15,000 μmol m^{-2} s^{-1} PFD of 0.8 s in duration) set to one flash per 100 s. The induction curves presented represent the mean of four replicate measurements using four different leaves.

The fluorescence parameter F_v/F_m was measured with a portable fluorometer (Plant Efficiency Analyser, Hansatech) to assess photoinhibition during high-irradiance experiments. Three replicate leaf discs were removed from the high irradiance after varying periods and dark-adapted for 30 min before the measurement of Chl *a* fluorescence. Quenching coefficients were also assessed using the fluorometer at the experimental irradiance and with the same intervals used for dark-adapted measurements.

Functional PSII: Oxygen Flash Yield

The number of functional PSII reaction centers capable of oxygen evolution was determined from the oxygen yield per single-turnover flash during repetitive (10 Hz) flash illumination, and expressed on a Chl basis (Chow et al., 1991). Four leaf discs (0.78 cm^2) were enclosed in a modified Hansatech leaf disc cuvette (model LD2, Hansatech) attached to a temperature-controlled water bath set at 23°C. Oxygen evolution was measured during a sequence of 4 min of dark followed by 4 min of repetitive flash illumination. This sequence was repeated twice and the rate of gross oxygen evolution in the light was calculated from the average of these two measurement intervals. The duration of the flash, measured as the full width at half-peak intensity, was approximately 2.5 μs. Background far-red light was used to avoid any limitation of electron transport by PSI. A small heating artifact resulting from the repetitive flash illumination was measured and subtracted from these calculations.

High Light Treatment

Leaf discs (0.78 cm^2) were punched from designated leaves and floated on distilled water, adaxial face up. Trays containing the leaf discs were then placed on a gently shaking platform in a temperature-controlled (20°C) water bath under a mercury vapor lamp (model HPLR, 1000 W, Philips, Cambridge, UK) with the light path interrupted by a glass heat and UV filter (Tempax 115, Schott, Cologne,

Table I. *Chl, PSII, and xanthophyll cycle pigment (V + A + Z) content of wild-type and ABA-deficient mutants of A. thaliana (aba1–3 and aba1–4) grown at 500 μmol m^{-2} s^{-1} PFD*

Leaves were collected 3 to 4 h into the photoperiod. Data represent the means ± SD of four to six different leaves.

Genotype	Chl	Chl *a/b*	PSII/Chl	(V + A) + Z	(V + A) + Z/Chl
	μmol m^{-2}		*mmol mol^{-1}*	*μmol m^{-2}*	*mmol mol^{-1}*
Wild type	359 ± 52	3.5 ± 0.1	2.8 ± 0.3	15.8 ± 0.6	45 ± 5
aba1–3	347 ± 16	3.7 ± 0.2	3.0 ± 0.2	48.2 ± 2.6	140 ± 7
aba1–4	375 ± 21	3.6 ± 0.1	2.6 ± 0.4	49.7 ± 2.4	133 ± 8

Germany). Leaf discs were exposed to an irradiance of 1000 μmol m^{-2} s^{-1} PFD for periods varying from 30 to 180 min.

RESULTS

Pigment Composition

Leaves and Thylakoids

The ABA-deficient mutants of Arabidopsis are unable to synthesize the epoxy-xanthophylls violaxanthin, neoxanthin, and antheraxanthin (Duckham et al., 1991; Rock and Zeevaart, 1991). Under the growth conditions described, the *aba1–3* and *aba1–4* mutants accumulated concentrations of xanthophyll cycle pigments (V + A) + Z, where V is violaxanthin, A is antheraxanthin, and Z is zeaxanthin, to a level 3-fold that of wild-type leaves (Table I). The increase in (V + A) + Z in the mutants was the result of pronounced zeaxanthin accumulation. The 3-fold increase in the ABA-deficient mutants was found in both whole-leaf extracts and washed thylakoid membranes, showing that the zeaxanthin was accumulating in photosynthetic membranes. However, approximately 35% of the (V + A) + Z pool was found in the chloroplast envelope in wild-type and mutant leaves (Table II). The existence of a significant carotenoid pool in the chloroplast envelope has been established previously (Siefermann-Harms et al., 1978; Douce and Joyard, 1979).

This increase in zeaxanthin in the ABA-deficient mutants occurred on a background of similar Chl content per unit area and similar PSII concentration per unit of Chl (Table I). However, the mutants did show a consistent (although minor) increase in the Chl *a/b* ratio (Table I) and a small decrease in lutein content compared with the wild-type plants (Table II), suggesting a smaller PSII light-harvesting antennae. Significantly, despite the 3-fold increase in xanthophyll cycle pigments in the mutants, the sum of (V + A + N) + Z, where N is neoxanthin, was only increased by approximately 30%, and the total amount of carotenoids was not changed in either of the mutants on a per unit of Chl basis compared with wild-type leaves (Table II). Our data suggest that the increase in zeaxanthin content in the ABA-deficient mutants occurs primarily as a one-for-one replacement of the missing violaxanthin and neoxanthin by zeaxanthin.

Thylakoid Pigment-Protein Complexes

The identity of the multiple Chl-protein complexes resolved from Arabidopsis was established by absorption spectroscopy of excised gel bands and by the polypeptide composition following SDS-PAGE (data not shown). These were similar to those described for barley thylakoids (Lee and Thornber, 1995). In order of increasing mobility the bands corresponded to the PSI-LHCI holocomplex, the PSI core reaction center, the PSII core reaction center, the major Chl *a/b*-binding pigment-protein complex LHCIIb, and the minor Chl *a/b*-binding pigment-protein complexes LHCIIa (CP29), LHCIIc (CP26), and LHCIId (CP24).

The disruption in carotenoid composition in the ABA mutants did not affect the partitioning of Chl to PSI and LHCI pigment-protein complexes (Table III). However, the absence of the epoxy-xanthophylls did reduce the partitioning of Chl to the major antenna complex of PSII (LHCIIb) in favor of the minor Chl *a/b*-binding proteins (LHCIIa, c, and d) of the inner antenna. This is consistent with the slightly higher Chl *a/b* ratio (Table I) and slightly

Table II. *Carotenoid content (per unit of Chl) of whole-leaf extracts and washed thylakoid membranes from wild-type and ABA-deficient mutants of A. thaliana (aba1–3 and aba1–4)*

Leaves were collected 3 to 4 h into the photoperiod. The data represent the means ± SD of four different leaves.

Carotenoid	Wild Type		*aba1–3*		*aba1–4*	
	Leaf	Thylakoid	Leaf	Thylakoid	Leaf	Thylakoid
			mmol mol^{-1}			
Violaxanthin	52 ± 7	33 ± 1	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Antheraxanthin	4 ± 1	2 ± 1	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Zeaxanthin	0 ± 0	0 ± 0	142 ± 2	93 ± 1	147 ± 20	89 ± 2
Neoxanthin	47 ± 3	42 ± 1	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Lutein	124 ± 3	107 ± 3	118 ± 6	78 ± 3	106 ± 18	91 ± 5
β-Carotene	120 ± 3	95 ± 1	130 ± 5	113 ± 2	132 ± 7	84 ± 6
(V + A) + Z	56 ± 6	35 ± 1	142 ± 2	93 ± 1	147 ± 20	89 ± 2
(V + A + N) + Z	103 ± 7	75 ± 1	142 ± 2	93 ± 1	147 ± 20	89 ± 2
Total carotenoid	347 ± 10	278 ± 5	390 ± 9	285 ± 4	385 ± 31	263 ± 7

Table III. *Relative Chl content (percentage) of the various pigment-protein complexes isolated from thylakoid membranes by Deriphat-PAGE*

The Chl content of the various pigment-protein bands resolved on the gel were determined by averaging the results of two scans at 675 and 650 nm. The identity of the different bands was established by absorption spectroscopy and by the polypeptide composition following SDS-PAGE. The Chl content of the PSII reaction center band could not be accurately separated from baseline variability, but it was determined to be less than 5%.

Genotype	PSI-LHCI	PSI Reaction Center	PSII Reaction Center	LHCIIb	LHCIIa, c, and d
Wild type	38.4	4.8	–[a]	42.5	10.9
aba1–3	44.7	4.7	–	30.7	17.4
aba1–4	43.3	5.5	–	31.5	16.7

[a] –, Less than 5%.

lower lutein content (Table II) found in the mutants, suggesting that there is a smaller PSII antenna in the ABA-deficient mutants.

In barley, LHCIIb units have been shown to contain one violaxanthin and two neoxanthin molecules per trimer (Lee and Thornber, 1995). Some of this violaxanthin is replaced by either antheraxanthin or zeaxanthin following exposure of the leaf to high irradiance (Lee and Thornber, 1995). Spinach LHCIIb trimers have also been reported to contain one violaxanthin, but only one neoxanthin molecule was found per trimer (Ruban et al., 1994). In this study we show that wild-type Arabidopsis LHCIIb also contains violaxanthin, neoxanthin, and lutein in an approximately 1:2:6 stoichiometry (Table IV). The LHCIIb complexes of the ABA-deficient mutants, however, show zea-xanthin:lutein ratios of 1:1.2 and 1:1.6 for *aba1–3* and *aba1–4*, respectively. This is lower than the 1:2 predicted by the wild-type data, suggesting some enrichment of zeaxanthin in LHCIIb in the mutants. The (V + A + N) + Z:lutein ratio in LHCIIa, c, and d was approximately 1:1 in the wild-type complexes and was unchanged in the two ABA-deficient mutants.

Using the relative Chl contents of these different pigment-protein complexes (Table III) we show that in wild-type leaves violaxanthin was partitioned, with 40% associated with PSI-LHCI, 30% with LHCIIb, and 27% with LHCIIa, c, and d (Tables III and IV). Therefore, 50% of the PSII-associated V + A + Z pigments is found with LHCIIb and 50% with LHCIIa, c, and d. On a per unit of Chl basis, this means that violaxanthin was particularly enriched in LHCIIa, c, and d (Table IV). In both *aba1–3* and *aba1–4*, zeaxanthin was partitioned, with 25% associated with PSI-LHCI, 36% associated with LHCIIb, and 37% associated with LHCIIa, c, and d. Thus, although more zeaxanthin was associated with PSII in the ABA-deficient mutants, zeaxanthin remained equally distributed between LHCIIb and LHCIIa, c, and d and was increased per unit of Chl in LHCIIa, c, and d (Tables III and IV).

Approximately 75% of the zeaxanthin pool was associated with PSII in the mutants, compared with 60% of violaxanthin in the wild-type complexes (Tables III and IV). If we apply this stoichiometry to the thylakoid pigment composition, the number of (V + A) + Z molecules per

Table IV. *Carotenoid composition (per unit of Chl) of the pigment-protein complexes separated by Deriphat-PAGE*

Pigmented bands were excised from eight replicate gels and the pigments were extracted from the polyacrylamide matrix by microelution. The pigment composition was then determined in 80% acetone by HPLC.

Carotenoid/Chl	Genotype	PSI-LHCI	PSI Reaction Center	PSII Reaction Center	LHCIIb	LHCIIa, c, and d
			mmol mol^{-1}			
Neoxanthin/Chl	Wild type	0	26	2	54	64
	aba1–3	0	0	0	0	0
	aba1–4	0	0	0	0	0
Violaxanthin/Chl	Wild type	33	26	5	23	81
	aba1–3	0	0	0	0	0
	aba1–4	0	0	0	0	0
Zeaxanthin/Chl	Wild type	0	0	0	0	0
	aba1–3	39	48	13	100	170
	aba1–4	49	41	16	81	164
(N + V) + Z/Chl	Wild type	33	52	7	77	145
	aba1–3	39	48	13	100	170
	aba1–4	49	41	16	81	164
Lutein/Chl	Wild type	46	72	70	167	192
	aba1–3	26	34	10	122	156
	aba1–4	48	38	20	126	172
β-Carotene/Chl	Wild type	143	129	126	12	35
	aba1–3	138	138	152	20	37
	aba1–4	114	114	104	15	42
Total carotenoid/Chl	Wild type	225	254	203	256	372
	aba1–3	205	224	175	243	364
	aba1–4	218	197	146	224	384

functional PSII, where PSII was assessed from oxygen flash yield measurements, increases from 8 in wild-type leaves to 23 and 26 for *aba1–3* and *aba1–4*, respectively (Tables I and II). However, the number of (V + A) + Z per functional PSII was 16, 23, and 26 for wild type, *aba1–3*, and *aba1–4*, respectively. The increase in (V + A + N) + Z per functional PSII in the ABA-deficient mutants was due to the slight enrichment of zeaxanthin in LHCIIb and in *aba1–4* to a lower concentration of functional PSIIs per Chl. These data show that, although (V + A) + Z increased 3-fold in the two ABA-deficient mutants, this increase can largely be explained as a one-for-one replacement by zeaxanthin of the missing neoxanthin and violaxanthin within the pigment-protein antennae complexes of both PSII and PSI.

Oxygen Evolution and Chl Fluorescence Quenching

The accumulation of a large pool of zeaxanthin in the two mutants did not reduce the apparent quantum yield or the light- and CO_2-saturated rate of photosynthetic oxygen evolution under continuous illumination (Fig. 1). Thus, for the ABA-deficient mutants, the altered xanthophyll composition has not affected their photosynthetic competence. We also found no differences between wild-type and ABA-deficient leaves in the light response of NPQ (Fig. 2A), PSII antenna trapping efficiency (Fig. 2B), PSII photochemical quenching (Fig. 2C), or the photochemical yield of PSII (Fig. 2D) measured in attached leaves in air. Similar results have been reported recently for the *aba1–1* mutant (Tardy and Havaux, 1996). The lack of any differences between wild-type and ABA-deficient mutant leaves in the way NPQ developed with increasing irradiance (Fig. 2A) was surprising, considering the larger pools of (V + A) + Z pigments in the two mutants and the fact that zeaxanthin was present in the mutant leaves at the beginning of the light curve measurements. In the ABA-deficient mutants, zeaxanthin seems active in quenching only when a pH gradient has been generated across the thylakoid membrane, and in the mutants NPQ appears to be determined by the strength of this gradient, not the high zeaxanthin concentration. Furthermore, the wild-type leaves can convert sufficient violaxanthin to zeaxanthin to generate an amount of NPQ equivalent to that of the mutants. Thus, the extra zeaxanthin in the mutants must not be active in quenching, although we have shown it is present within the pigment-protein complexes and almost certainly at the sites normally occupied by violaxanthin and neoxanthin.

The only differences that could be found were during dark-to-light transitions, in which the mutant leaves developed NPQ more rapidly and reached a higher peak during the first 2 min of illumination. We show this both at the growth irradiance (500 μmol m^{-2} s^{-1} PFD) and at saturating irradiance (1000 μmol m^{-2} s^{-1} PFD; Fig. 3, B and D, respectively). However, this change in the initial develop-

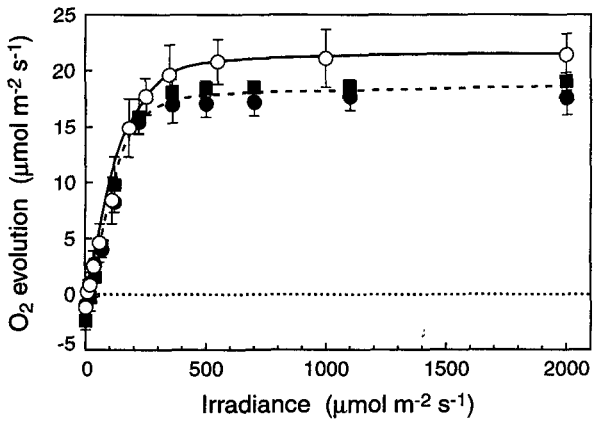

Figure 1. Irradiance-response curves of CO_2-saturated photosynthetic oxygen evolution measured on detached leaf discs at 23°C. O, Wild type; ●, *aba1–3*; and ■, *aba1–4*. Each point represents the mean ± SD of four different leaves. Where error bars are missing, error was smaller than the symbol size.

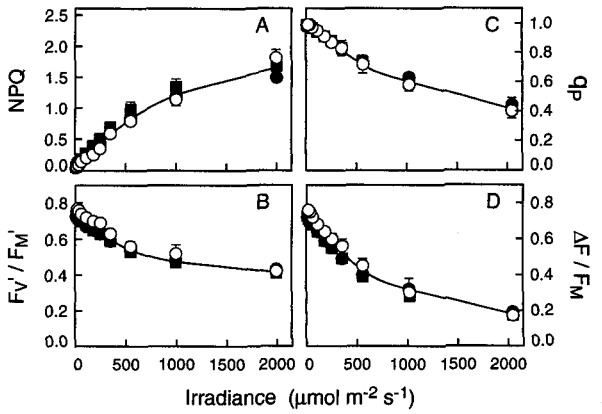

Figure 2. Irradiance-response curves of Chl a fluorescence parameters measured on attached leaves in air at 23°C. (O), Wild type; ●, *aba1–3*; and ■, *aba1–4*. The initial F_v/F_m for these leaves were 0.79 ± 0.02, 0.76 ± 0.01, and 0.76 ± 0.01 for wild type, *aba1–3*, and *aba1–4*, respectively. Where error bars are missing, error was smaller than the symbol size. Each point represents the mean ± SD of four different leaves.

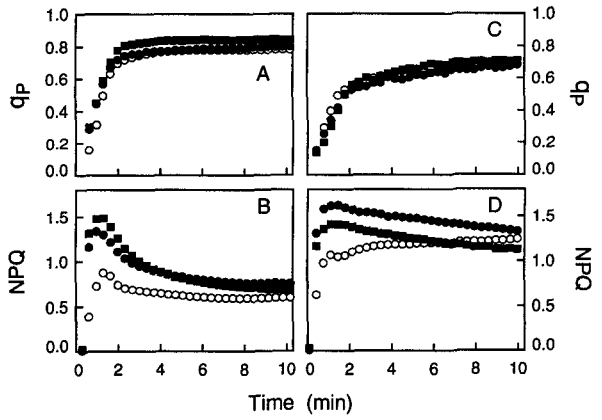

Figure 3. Comparison of the time courses of photochemical (A and C) and nonphotochemical (B and D) fluorescence quenching induction following a shift from darkness to 500 μmol m^{-2} s^{-1} PFD (A and B) or 1000 μmol m^{-2} s^{-1} PFD (C and D). O, Wild type; ●, *aba1–3*; and ■, *aba1–4*. Measurements were made on attached leaves in air at 23°C. Each point represents the mean of four different leaves.

ment of NPQ did not affect the development of q_P (Fig. 3, A and B), and when steady-state fluorescence was established after 4 to 6 min of illumination there were no differences between the wild-type and mutant leaves (Fig. 3, B and D). Furthermore, as shown by Pesaresi et al. (1995), once both wild-type and mutant leaves reach steady-state photosynthesis, subsequent changes in irradiance do not lead to more rapid changes in NPQ in the mutants. Thus, it is likely that this small window represents the time lag required for fully dark-adapted wild-type leaves to convert some violaxanthin to zeaxanthin rather than a general reduction in the quenching response time of the mutants.

Response of the ABA-Deficient Mutants to High Irradiance

We used the decline in Chl *a* fluorescence to quantify photoinhibition during prolonged exposure to high irradiance. However, because of the pronounced changes in carotenoid composition in the ABA-deficient mutants, and therefore the potential for altered dark-adapted Chl *a* fluorescence quenching characteristics, we also used oxygen yield during repetitive single turnover flashes to quantify the number of functional PSII complexes in leaves (Chow et al., 1991). A linear relation was found in leaves of wild-type and ABA-deficient mutants between changes in dark-adapted F_v/F_m and the number of functional PSII complexes measured by oxygen flash yield without a dark period (Fig. 4). Thus, both methods reflect similar changes in PSII functionality in the wild-type and ABA-deficient mutants.

The sensitivity of wild-type Arabidopsis and the two ABA-deficient mutants to photoinhibition was tested by exposing leaf discs to a 2-fold increase in irradiance (1000 μmol m^{-2} s^{-1} PFD) for periods of up to 180 min (Fig. 5). This treatment resulted in a q_P of approximately 0.5 in both wild-type and mutant leaves, although q_P increased slightly in all leaves with time (Fig. 5A). Wild-type and ABA-deficient mutant leaves maintained similar PSII antenna-trapping efficiencies throughout the high irradiance treatment (F_v'/F_m') (Fig. 5B). However, when we calculated the product of these two parameters ($\Delta F/F_m$), we showed that the photochemical yield was slightly higher in both mutants throughout the high irradiance treatment (Fig. 5C), reflecting minor differences in q_P (Fig. 5A). Surprisingly, the maximum NPQ that developed with prolonged exposure to high irradiance was greater in the wild-type leaves than in either of the ABA-deficient mutants (Fig. 5F), despite the much larger pools of zeaxanthin in the mutants (Fig. 5D). This suggests that, although zeaxanthin replaced the violaxanthin and neoxanthin within the pigment-protein complexes, the loss of these epoxy-xanthophylls limits quenching capacity during prolonged high-light stress.

No differences were found in in vivo photoinhibition, measured either as dark-adapted F_v/F_m or functional PSII from oxygen flash yield measurements (Fig. 5, D or E, respectively). When we consider the much greater zeaxanthin content of the mutant leaves, which we have shown to be present in the thylakoid (Table II) and to be associated with the pigment-protein complexes (Table IV), the lack of any increased NPQ or photoprotective benefit from this zeaxanthin was surprising. Recently, leaves of the *aba1–1*

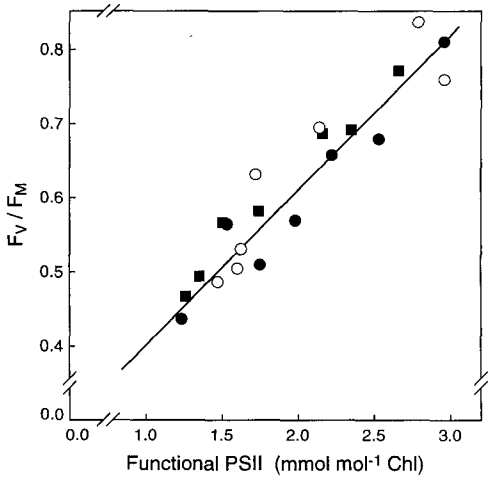

Figure 4. Relationship between changes in functional PSII as determined by oxygen flash yield and dark-adapted F_v/F_m. ○, Wild type; ●, *aba1–3*; and ■, *aba1–4*.

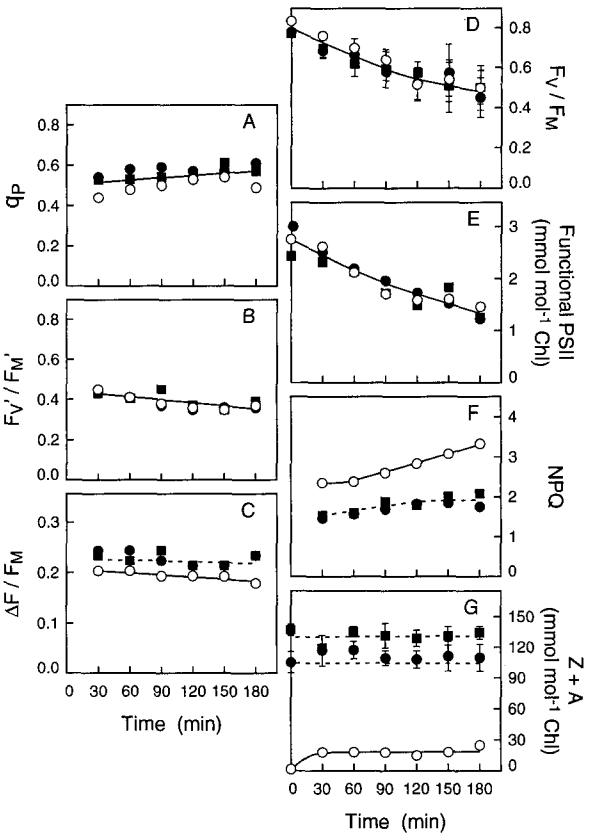

Figure 5. Response of Chl *a* fluorescence quenching, PSII function, and violaxanthin de-epoxidation in wild-type (○), *aba1–3* (●), and *aba1–4* (■) leaves to exposure to an irradiance of 1000 μmol m^{-2} s^{-1} PFD. Each point in the fluorescence quenching curves represents the mean ± SD of three replicate leaf discs. For F_v/F_m, n = 7, and for functional PSII, n = 4. Where error bars are missing, error was smaller than the symbol size.

mutant, which also accumulates large pools of zeaxanthin, were shown to be as sensitive to photoinhibition as wild-type leaves (Tardy and Havaux, 1996). These data strongly suggest that not all zeaxanthin produced by violaxanthin de-epoxidation can be involved in energy dissipation in the antenna of PSII.

DISCUSSION

The ABA-deficient mutants of Arabidopsis (*aba1–3* and *aba1–4*) are unable to synthesize detectable amounts of the epoxy-xanthophylls neoxanthin, antheraxanthin, and violaxanthin and instead accumulate zeaxanthin (Table I; Duckham et al., 1991; Rock and Zeevaart, 1991). The primary lesion in these ABA-deficient mutants is the loss of zeaxanthin epoxidase (Marin et al., 1996). The relative distribution of the xanthophyll cycle pigments was 40 and 25% in PSI-LHCI, 30 and 36% in the major Chl *a/b*-binding protein (LHCIIb), and 27 and 37% in the minor Chl *a/b*-binding proteins (LHCIIa, c, and d) in wild-type and *aba1–3* and *aba1–4* leaves, respectively. Thus, in Arabidopsis approximately one-third of all V + A + Z pigments are partitioned to PSI-LHCI, and, of the remaining two-thirds associated with PSII, 50% is partitioned to LHCIIb and 50% to LHCIIa, c, and d. The distribution of these pigments is currently under dispute in the literature, with reports varying from 80% of the PSII-associated V + A + Z partitioned to LHCIIa, c, and d in *Zea mays* (Bassi et al., 1993) to 50% in spinach (Ruban et al., 1994). Whether these differences reflect real variation between species or the different solubilization procedures is not yet clear.

It has previously been shown that the assembly of functional LHCII complexes in vitro requires the presence of violaxanthin or neoxanthin (Plumbley and Schmidt, 1987). However, Rock et al. (1992) showed similar LHCII abundance in wild-type and the *aba1–4* mutant, suggesting similar LHCII protein assembly and accumulation in this violaxanthin- and neoxanthin-deficient mutant. In a more detailed study, Pesaresi et al. (1995) also reported only minor changes in the concentration of the individual pigment-protein complexes in the *aba1–3* mutant. When we compare the concentration of (V + A + N) + Z in the various complexes of the wild-type and mutant leaves, zeaxanthin apparently replaces the missing violaxanthin and neoxanthin within both major and minor Chl *a/b*-binding complexes of PSII, and within PSI-LHCI, in these ABA-deficient mutants. These data extend earlier preliminary reports of the *aba1–3* and *aba1–4* mutants (Hurry, 1995; Pesaresi et al., 1995) and suggest that there are binding sites within the pigment-protein complexes that can be occupied by either violaxanthin, antheraxanthin, neoxanthin, or zeaxanthin. This could account for the puzzling lack of integral stoichiometry of the epoxy-xanthophylls found in particular pigment-protein complexes. Thus, aside from the two luteins per LHCIIb monomer needed for structural stability (Kühlbrandt et al., 1994), at least one other of these additional xanthophylls (violaxanthin, antheraxanthin, neoxanthin, or zeaxanthin) may be required for the assembly of functional LHCII complexes in vivo. In the inner antenna (LHCIIa, c, and d), this stoichiometry appears to increase to one violaxanthin, antheraxanthin, neoxanthin, or zeaxanthin molecule per lutein. However, in both mutants we found a small reduction in Chl and lutein partitioned to LHCIIb. These data suggest that, although zeaxanthin can replace both violaxanthin and neoxanthin in the assembly of functional LHCII complexes in vivo, either neoxanthin or violaxanthin may play a minor structural or stabilizing role in the LHCIIb pigment-protein complex that zeaxanthin is unable to complement exactly.

During prolonged exposure to high irradiance we observed a reduction in the capacity of both mutants to develop NPQ, which suggests that there is a role for either violaxanthin or neoxanthin in nonradiative energy dissipation. Alternatively, the correlation between the slight loss of LHCIIb and the lower NPQ suggests an important role for the distal antenna complexes in the development of maximal NPQ. This supports the hypothesis that LHCIIb plays a major role in antenna quenching (Ruban et al., 1992; Ruban and Horton, 1995). However, recent findings from the Chl *b*-deficient *chlorina* barley mutants show that both the intensity of antenna quenching and the Chl *a* fluorescence lifetime distributions were independent of the size of the distal LHCIIb antenna (Gilmore et al., 1996b). The conclusion drawn from these studies was that the LHCIIb complexes do not participate in NPQ reactions. Unfortunately, the data reported here do not help resolve this question because we cannot eliminate the possibility that in the mutants minor structural changes in the antenna may have resulted from the substitution of zeaxanthin for the epoxy-xanthophylls and that this may have limited the development of maximal NPQ. Nevertheless, our data show that the epoxy-xanthophylls (either neoxanthin or violaxanthin) do play a role in the development of maximal NPQ in vivo and that replacing all of the violaxanthin in the antennae complexes with zeaxanthin does not necessarily increase nonphotochemical energy dissipation.

We have shown that the (V + A) + Z content per PSII increases from approximately 8 in the wild-type leaves to 23 and 26 in the *aba1–3* and *aba1–4* mutants, respectively. This change in (V + A) + Z has not affected the relative distribution of the major and minor Chl *a/b*-binding proteins in wild-type and the ABA-deficient mutants, as judged by Chl content. Therefore, one would expect that the organization of the Chl *a/b*-binding protein complexes in vivo is also similar. This increase in zeaxanthin associated with the pigment-protein complexes of the ABA-deficient mutants did not lead to increased NPQ, nor did it result in any change in the sensitivity of the mutant leaves to photoinhibition. Furthermore, the substitution of zeaxanthin for neoxanthin and violaxanthin within the pigment-protein complexes of the ABA-deficient mutants, without any increase in quenching capacity, clearly shows that not all bound zeaxanthin can function to quench excitation energy. Thus, our data strongly suggest that the existence of violaxanthin that can undergo de-epoxidation to zeaxanthin in the thylakoid does not necessarily mean that the zeaxanthin produced will be active in energy dissipation and photoprotection, in agreement with the hy-

pothesis of Crofts and Yerkes (1994). This is not a particularly surprising conclusion if we consider that in Arabidopsis 40% of the zeaxanthin is associated with PSI. Moreover, a significant fraction (30%) is also associated with LHCIIb, and if LHCIIb does not participate in NPQ (Gilmore et al., 1996b), this will also reduce the pool of "active" zeaxanthin. Gilmore et al. (1996b) recently proposed a model suggesting that xanthophyll cycle-dependent fluorescence quenching is determined by both the strength of the pH gradient and the binding frequency of Z + A to specific sites within the inner PSII antenna (LHCIIa, c, and d). Thus, increases in xanthophyll cycle-related energy dissipation will not be linearly related to the concentration of Z + A in the thylakoid. Rather, if the switching of a "normal" PSII center to a "quenching" center is dependent on the binding frequency of Z + A at specific sites, this binding interaction will show curvilinear saturation kinetics. However, an essentially linear relation may be found between antenna quenching and Z + A concentration during the initial phase of conversion of V to A + Z.

In summary, we have shown that zeaxanthin can completely replace the neoxanthin and violaxanthin normally present in both the minor and major Chl *a*/*b*-binding pigment-protein complexes of PSII and within PSI-LHCI without significantly altering photosynthetic performance or greatly affecting antennae composition or Chl fluorescence quenching characteristics. This suggests that these xanthophylls do not occupy specific binding sites but that general binding sites can be occupied by either violaxanthin, antheraxanthin, neoxanthin, or zeaxanthin. However, the fact that violaxanthin and neoxanthin are replaced by stoichiometric amounts of zeaxanthin in the antenna complexes of the two ABA-deficient mutants shows that these xanthophylls are required in addition to lutein for the proper assembly of functional LHCII and LHCI complexes in vivo. We also show that light-dependent conversion of violaxanthin to antheraxanthin and zeaxanthin is not required for the development of NPQ and that zeaxanthin present in the dark can function. However, the generation of a pH gradient is required for NPQ, even in plants containing large dark pools of zeaxanthin. These data strongly suggest that only a few of the zeaxanthin molecules present within the light-harvesting antenna of PSII may act in the development of NPQ and that the accumulation of large thylakoid xanthophyll pools or the greater conversion of violaxanthin to zeaxanthin will not necessarily enhance photoprotective energy dissipation.

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